

F-Spondin Is Required for Accurate Pathfinding of Commissural Axons at the Floor Plate

Tal Burstyn-Cohen,* Vered Tzarfaty,* Ayala Frumkin,* Yael Feinstein,* Esther Stoeckli,[†] and Avihu Klar*[‡]

*Department of Anatomy and Cell Biology
The Hebrew University
Hadassah Medical School
Jerusalem 91120
Israel

[†]Department of Integrative Biology
University of Basel
Rheinsprung 9
CH-4051 Basel
Switzerland

Summary

The commissural axons project toward and across the floor plate. They then turn into the longitudinal axis, extending along the contralateral side of the floor plate. F-spondin, a protein produced and secreted by the floor plate, promotes adhesion and neurite extension of commissural neurons in vitro. Injection of purified F-spondin protein into the lumen of the spinal cord of chicken embryos in ovo resulted in longitudinal turning of commissural axons before reaching the floor plate, whereas neutralizing antibody (Ab) injections caused lateral turning at the contralateral floor plate boundary. These combined in vitro and in vivo results suggest that F-spondin is required to prevent the lateral drifting of the commissural axons after having crossed the floor plate.

Introduction

The immense diversity of neuronal cell types and the formation of specific connections between distinct subsets of neurons are critical for the generation of functional neural circuits. Recent studies have shown that guidance molecules can guide axons either by promoting or inhibiting outgrowth. These outgrowth modulators can act either as short-range cues in the form of membrane-attached and extracellular matrix-bound proteins or as long-range cues in the form of diffusible molecules. It is the relative balance between attractive and repulsive forces that regulates the directionality of axonal outgrowth during development (Tessier-Lavigne and Goodman, 1996). Growing axons negotiate their way to their targets by continuously sampling their environment for guidance cues. Thus, growth cone guidance can be viewed as an infinite series of integration of diverse signals derived from multiple molecular interactions at each site along the axon's pathway.

The trajectory of the commissural axons in the embryonic spinal cord is a well-studied model system for axonal pathfinding. The commissural neurons originate in the dorsal spinal cord and project their axons ventrally toward the ventral midline. After crossing the ventral

midline (consisting of specialized floor plate cells), the commissural axons change their trajectory from the transverse plane to the longitudinal axis (Holley and Silver, 1987; Dodd and Jessell, 1988; Bovolenta and Dodd, 1990).

A role for both the dorsal midline cells (the roof plate) and the ventral midline cells (the floor plate) in the guidance of commissural axons has been demonstrated (Tessier-Lavigne et al., 1988; A. Augsburger et al., 1996, Soc. Neurosci., abstract). In corroboration, embryonic mutations, which result in the absence of the floor plate, lead to errors in the pathfinding of commissural axons (Bovolenta and Dodd, 1991; Bernhardt et al., 1992; Hatta, 1992).

The initial trajectory of commissural axons is determined both by chemoattraction and chemorepulsion (Tessier-Lavigne et al., 1988). The intermediate target for these axons, the floor plate, produces and secretes netrin-1, which acts as a long-range chemoattractant (Kennedy et al., 1994; Serafini et al., 1994). Deleted in Colorectal Cancer (DCC), which is a member of the immunoglobulin (Ig) superfamily of cell adhesion molecules (CAMs) and is expressed by commissural axons, has been identified as a receptor for netrin, mediating its attractive activity (Keino-Masu et al., 1996; Kolodziej et al., 1996; Fazeli et al., 1997).

The trajectory of commissural axons across the floor plate is determined by a balance between positive and negative signals (Seeger et al., 1993; Stoeckli et al., 1997). Positive signals, which are required at the ipsilateral side of the floor plate, are derived from interactions between Ig superfamily CAMs on the floor plate surface and on the surface of commissural growth cones (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997). Perturbation of axonin-1 and NrCAM interactions resulted in pathfinding errors of the commissural axons. In the absence of axonin-1 and NrCAM interactions, some commissural axons failed to cross the midline and erroneously turned prematurely into the longitudinal axis along the ipsilateral floor plate border.

At the contralateral side of the floor plate, commissural axons are expelled from the floor plate and are prevented from recrossing the midline. Genetic evidence in *Drosophila* and *Caenorhabditis elegans* demonstrates that this function is mediated by an upregulation of Roundabout (Robo) (Seeger et al., 1993; Kidd et al., 1998a, 1998b; Zallen and Bargmann, 1998), a receptor for Slit. Slit's inhibitory activity was shown to be associated with the midline (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999). Robo is upregulated in the commissural axons only after they have crossed the midline, whereas axons projecting ipsilaterally express Robo from the onset of axon elongation (Kidd et al., 1998b). Another dynamic process of commissural axons is the loss of netrin responsiveness after having crossed the floor plate (Shirasaki et al., 1998).

It is not yet clear what determines the sharp rostral turn of the commissural axons extending in close contact with the contralateral floor plate boundary. Two hypotheses may account for a mechanism that prevents the lateral drifting of the commissural axons from the

[‡]To whom correspondence should be addressed (e-mail: avihu@cc.huji.ac.il).

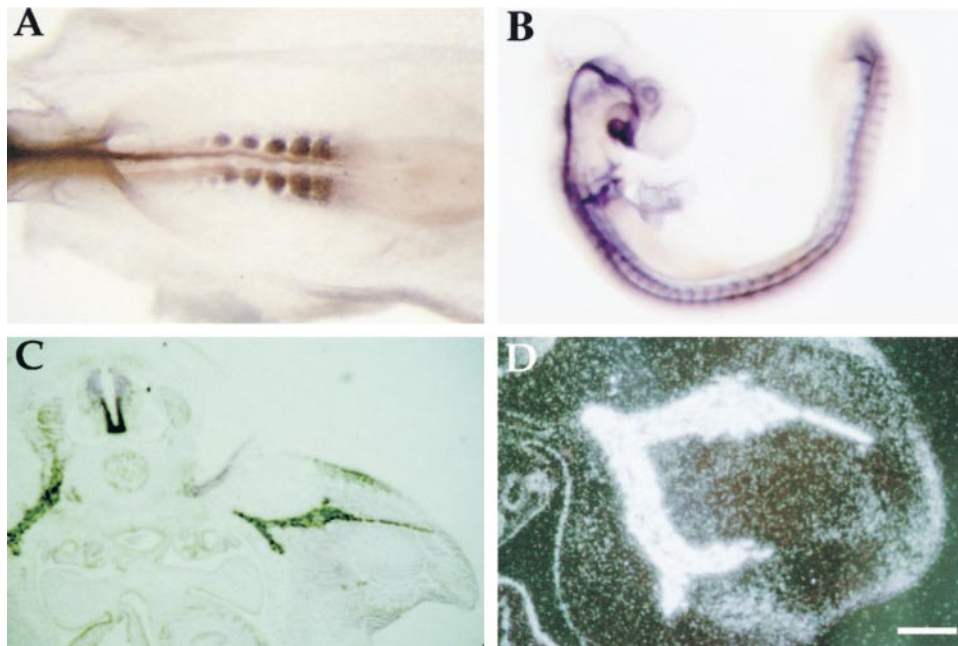


Figure 1. Localization of *F-Spondin* mRNA in Chick Embryo

F-spondin mRNA was localized with digoxigenin-labeled (A and B) or radiolabeled (C and D) antisense probes.

(A) At stage 8 Hamburger–Hamilton (E2), *F-spondin* mRNA is detected in the notochord and the somites. Rostral is to the left. Scale bar, 200 μ m.

(B) At E3, high levels of *F-spondin* transcript are detected in the floor plate, the retina, the notochord, the caudal part of the somites, the heart, and along the cranial nerves. Scale bar, 400 μ m.

(C) Bright-field image of a cross section of an E5 chick embryo, displaying *F-spondin* transcripts in the floor plate, ventral ventricular zone, notochord, and dorsal root ganglia as well as along the peripheral nerve. Scale bar, 100 μ m.

(D) Dark field image of the limb bud of an E5 chick embryo, exhibiting high levels of expression along the peripheral nerve. Scale bar, 50 μ m. Scale bar values given for bar shown in (D).

contralateral floor plate boundary. First, repulsive factors from opposite directions, namely the floor plate and the lateral spinal cord epithelium, prevent the lateral drifting. Thus, two repulsive cues are “pushing” the axons, thereby enforcing the growth along the contralateral floor plate boundary—one force emanating from the floor plate, pushing laterally (i.e., Slit), and another emanating from the lateral neural epithelium and pushing toward the floor plate. Second, attractive factors emanating from the floor plate may also play a role. The floor plate might secrete short-range attractants that “pull” the axons back to the floor plate. Thus, both the push activity provided by Slit and the pull activity provided by some other putative attractants might be required for keeping the axons along the floor plate contralateral boundary.

We have previously isolated a floor plate gene, *F-spondin*, which encodes an extracellular matrix (ECM) protein with adhesive properties (Klar et al., 1992a, 1992b). *F-spondin* encodes a secreted molecule of 807 amino acids. The carboxy-terminal half of the protein (440–807) contains six thrombospondin type 1 repeats (TSR) (Lawler and Hynes, 1986; Bornstein et al., 1991). The amino-terminal half is composed of two domains: amino acids 1–200 share homology with the amino-terminal portion of reelin, a protein implicated in guiding the migration of cortical neuroblasts (D’Arcangelo et al., 1995); amino acids 200–440 form the spondin domain. This domain characterizes a new protein family, the minidins. They are secreted molecules that bind to the ECM

containing the spondin domain as well as a TSR domain (Higashijima et al., 1997; Umemiya et al., 1997; Y. F. et al., unpublished data).

Recombinant *F-spondin* promotes neural cell adhesion and neurite extension of dorsal root ganglia (Klar et al., 1992a; Burstyn-Cohen et al., 1998) and hippocampal neurons (Y. F. et al., unpublished data) in vitro. Thus, both its activity and its pattern of expression suggest that secreted *F-spondin* might play a role in guidance of the commissural axons at the floor plate.

In the current study, we show that the *F-spondin* protein promotes neural cell adhesion and neurite extension of commissural neurons in vitro. Immunohistochemical analysis with anti-*F-spondin* antibodies (Abs) demonstrates that the protein is localized in the basal lamina underlying the floor plate. In vivo perturbations of *F-spondin* by injection of either purified *F-spondin* protein or Abs into the central canal of the spinal cord of chicken embryos in ovo caused pathfinding errors of commissural axons. These results suggest that *F-spondin* plays an important role in the guidance of commissural axons at the floor plate.

Results

Expression Pattern of Chick *F-Spondin*

F-spondin, a floor plate gene encoding a secreted, matrix-attached adhesion molecule, was previously identified in rodents (Klar et al., 1992a), *Xenopus* (Klar et al.,

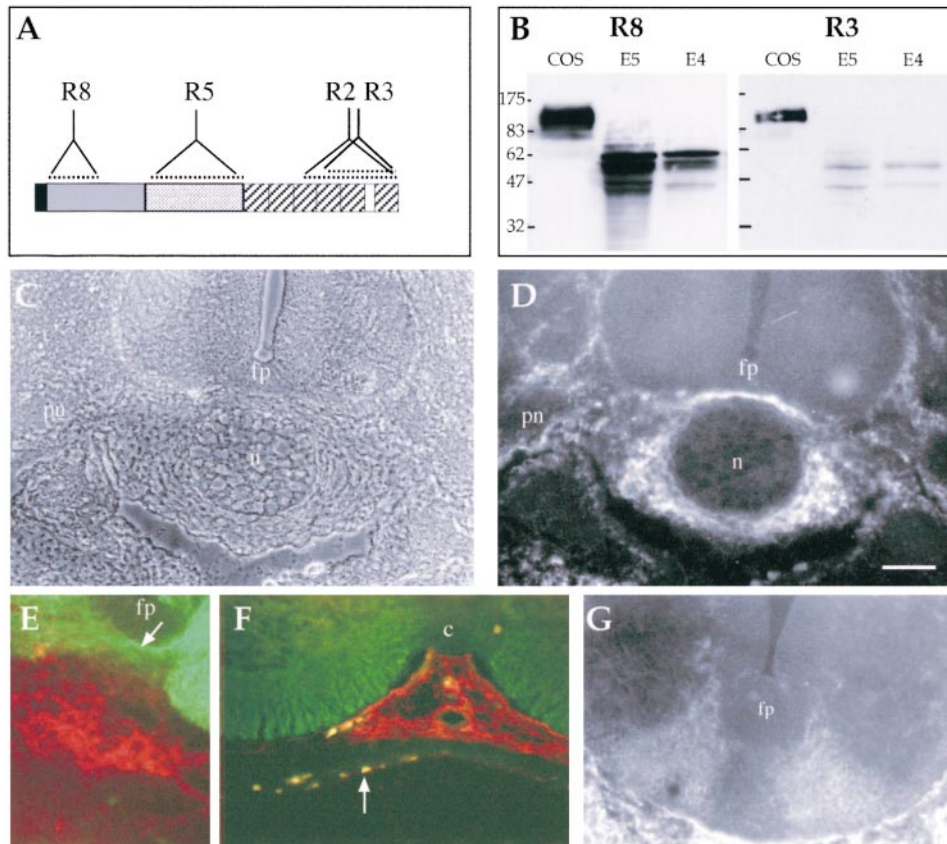


Figure 2. F-Spondin Protein Is Processed and Deposited in the Basal Membrane that Underlies the Floor Plate

(A) Schematic representation of the F-spondin domain structure. The closed box represents the signal sequence; the shaded box, the reelin domain; the dotted box, the spondin domain; the hatched boxes, the TSRs. The broken lines represent the regions used to generate the Abs. (B) Western blot analysis of the F-spondin protein. A 115 kDa protein is detected in the conditioned media of transfected COS cells with both the R8 and the R3 Abs. A 60 kDa protein corresponding to the reespo domain is detected with the R8 Ab in protein extract of E5 and E4 chick spinal cords. Proteins (50 and 40 kDa) corresponding to the TSR domain are detected with the R3 Ab in protein extracts of E5 and E4 chick spinal cords. (C) Phase-contrast image of a cross section of an E4 chick embryo. Scale bar, 50 μ m. (D) Immunolocalization of F-spondin protein with the R5 Ab (same section as in [C]). The protein is localized in the basal membrane that underlies the floor plate, around the notochord and in the basal membrane that ensheaths the peripheral nerve. Scale bar, 50 μ m. (E) E14 rat ventral spinal cord immunolabeled with R5 Ab (TRITC) and HNK-1 mAb (FITC). F-spondin protein accumulates under the floor plate cells. The commissural axons (arrow) are in contact with the F-spondin protein. Scale bar, 25 μ m. (F) E16 ventral spinal cord immunolabeled with R5 Ab (TRITC) and HNK-1 mAb (FITC). At this stage, the HNK-1 labels only the neuroepithelium. F-spondin protein accumulates under the floor plate cells, above the pial surface (arrow). Scale bar, 20 μ m. (G) Immunolocalization of F-spondin protein in the midbrain region. The protein spreads laterally. Abbreviations: n, notochord; fp, floor plate; and pn, peripheral nerve. Scale bar, 25 μ m. Scale bar values given for bar shown in (D).

1992b; Ruiz i Altaba et al., 1993), and zebrafish (Higashijima et al., 1997). F-spondin may be implicated in commissural axon guidance in the following ways: first, F-spondin may act as a diffusible chemoattractant, guiding the commissural axons toward the floor plate; second, it may act as a contact-mediated attractant molecule required for crossing the floor plate; third, it may act as a contact-mediated repellent molecule preventing the recrossing of the floor plate; fourth, it may act as a short-range attractant, either chemo- or contact, reattracting the axons to the floor plate at the contralateral boundary.

To investigate the role of F-spondin, we have cloned the avian homolog and studied its function in chick embryos, since this system can be manipulated by introduction of either mislocalized ectopic protein or blocking Abs in ovo. To clone chick F-spondin, a cDNA library

of embryonic day 2.5 (E2.5) spinal cord was screened with rat *F-spondin* cDNA as a probe. Several clones were identified, one of which contained an insert of 4 kb. Translation of the open reading frame of *cF-spondin* predicted a protein of 802 amino acids. The hydrophobic signal sequence at the amino terminus is followed by a reelin domain (amino acids 20–200), a spondin domain (amino acids 200–425), and six TSRs (TSR domain, amino acids 425–802) (Figure 2A). The predicted size and domain organization of chick F-spondin are identical to those of rat, *Xenopus*, and zebrafish F-spondins.

To determine the spatial and temporal distribution patterns of *F-spondin* mRNA during avian embryogenesis, we performed in situ hybridization analyses in chick (Figure 1). The first F-spondin transcripts were found to coincide with the onset of somite formation around

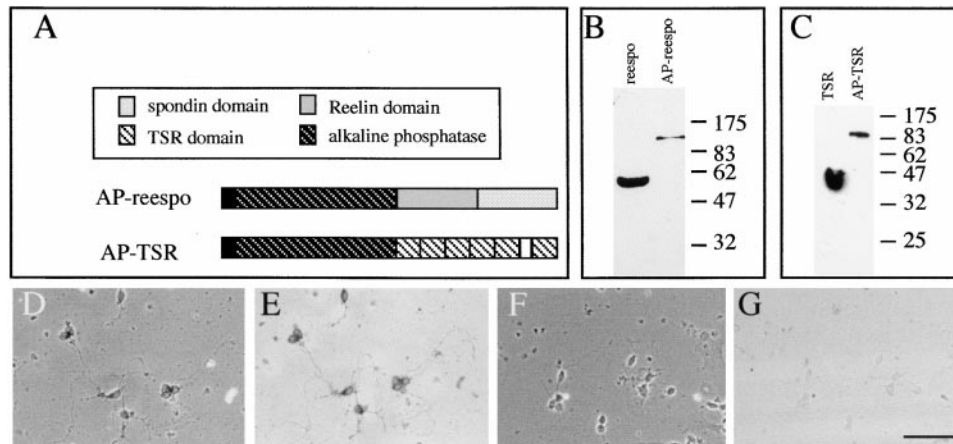


Figure 3. The TSR Domain Binds to Commissural Neurons

(A) Schematic representation of the expression vectors of F-spondin domain fusion proteins. The closed box represents the signal sequence. The dotted box represents the spondin domain. The hatched boxes represent the TSRs. The shaded box represents the reelin domain. The negative hatched box represents the AP gene.
 (B) Western blot with the R5 Ab of conditioned media of HEK 293 cells transfected with the reespo domain protein (left) and AP-reespo fusion protein (right). Bands of 60 kDa and 120 kDa, respectively, are detected.
 (C) Western blot with the R2 Ab of conditioned media of HEK 293 cells transfected with the TSR domain protein (left) and AP-TSR fusion protein (right). Bands of 40 kDa and 110 kDa, respectively, are detected.
 (D) Phase-contrast image of commissural neurons incubated with AP-TSR.
 (E) Bright-field image of (D) showing AP reactivity on the cell bodies and along the axons.
 (F) Phase-contrast image of commissural neurons incubated with AP-reespo.
 (G) Bright-field image of (F) showing no AP reactivity.
 Scale bar, 100 μ m (D–G).

stage 7 (Hamburger and Hamilton, 1951; Debby-Brafman et al., 1999) (Figure 1A). During the early stages of somitogenesis, *F-spondin* mRNA expression appeared homogeneously distributed throughout the extent of the 9–11 rostralmost somite pairs and in the notochord. At later stages, each somite pair that had segregated from the paraxial mesoderm clearly revealed a more prominent F-spondin signal in its caudal part (Debby-Brafman et al., 1999) (Figure 1B). Expression of F-spondin in the floor plate was apparent at stage 16. The expression became more abundant as development proceeded (E3–E9; E9, oldest age checked) (Figures 1B and 1C). At E5, the expression of F-spondin expanded dorsally; the ventral ventricular zone immediately adjacent to the floor plate began to express high levels of *F-spondin* mRNA (Figure 1C). In addition to the expression of F-spondin in the embryonic CNS, after E4, hybridization was also detected in association with sensory and motor nerve branches that project into the periphery (Figures 1C and 1D). Thus, like in rodents, F-spondin expression in the nervous system is predominantly found in the floor plate, the ventral ventricular zone, and early Schwann cells. By contrast, expression in the early somites is unique to the avian gene. In rodents, somitic F-spondin expression was observed only in older embryos (E12) in the caudal somites (data not shown).

F-Spondin Protein Is Localized to the ECM that Underlies the Floor Plate

We have previously generated domain-specific Abs against rat F-spondin (Burstyn-Cohen et al., 1998). Western blot analysis of transfected COS cells revealed

that a spondin domain-specific Ab (R5) and a TSR domain-specific Ab (R2) recognized the chick protein (data not shown). Two additional Abs were raised, one against the reelin domain of the rat F-spondin (amino acids 27–130; R8), and one against TSRs 4–6 of the chick F-spondin (R3) (Figure 2A). The R8 and R3 Abs recognized a 115 kDa protein in the conditioned media of transfected COS cells (Figure 2B), identical to the size of the protein recognized by the R2 and R5 Abs (Burstyn-Cohen et al., 1998). Western blot analysis of the protein extracts of chick spinal cord revealed a 60 kDa protein recognized by the R8 and R5 Abs as well as one 50 and one 40 kDa protein recognized by the R3 and R2 Abs (Figure 2B; R2 and R5, data not shown). Bands of similar molecular weight were also detected in a peripheral nerve protein extract (Burstyn-Cohen et al., 1998). The size of the protein bands and their recognition by domain-specific Abs imply that F-spondin is proteolytically processed in vivo to generate a reelin/spondin (reespo) fragment of 60 kDa, a fragment comprising 6 TSRs of 50 kDa, and a fragment of 5 TSRs of 40 kDa.

Taken together, the timing of expression, the mRNA localization, and the outgrowth-promoting activity of F-spondin (Klar et al., 1992a; Burstyn-Cohen et al., 1998) implicate the protein in the guidance of commissural axons. It is expected that floor plate proteins involved in attracting commissural axons to the ventral midline form a ventral-to-dorsal gradient. On the other hand, proteins that guide commissural axons at the floor plate, either across it or along its contralateral border, are expected to be presented more locally, either on the floor plate cells themselves or in the basal membrane

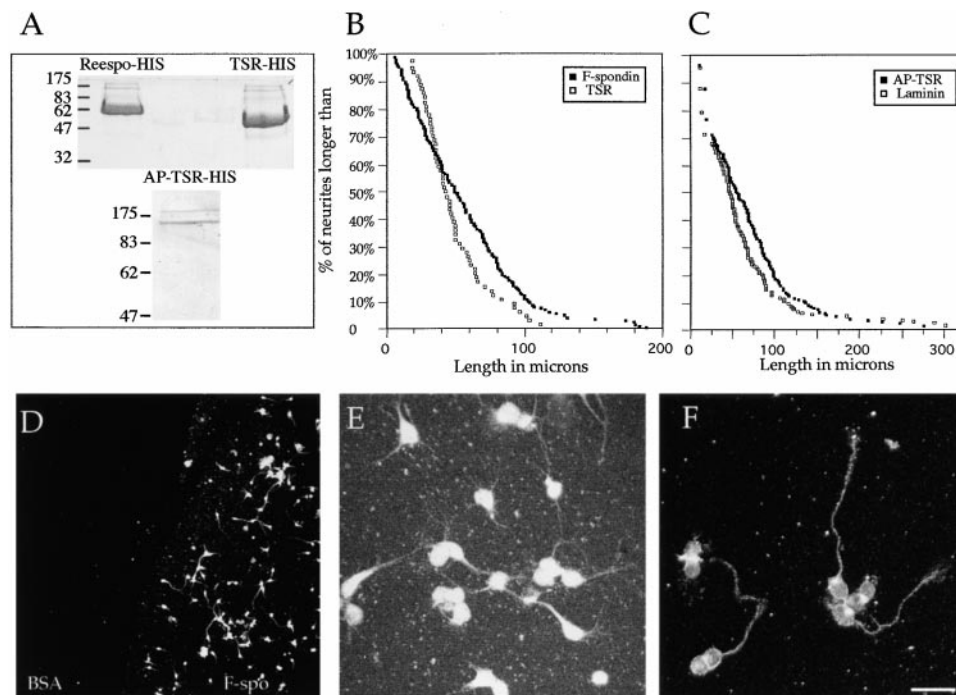


Figure 4. The TSR Domain Protein Promotes In Vitro Outgrowth of Commissural Neurons

(A) Reespo-HIS, TSR-HIS, and AP-TSR-HIS proteins obtained from affinity-purified medium of transfected HEK 293 cells. A single band is observed on an SDS-PAGE gel stained with Coomassie blue.
 (B) Outgrowth of rat E13 commissural neurons on a substrate of F-spondin-HIS and TSR-HIS. Purified proteins (40 μ g/ml) were immobilized onto a nitrocellulose monolayer as described (Lemmon et al., 1989). The neurons were stained with the anti-tubulin III mAb (TUJ-1). For each TUJ-1-positive neuron, the neurite length was measured, or if no neurite was seen, considered to be 0. The percentage of neurons (ordinate) with neurites longer than a given length (in micrometers; abscissa) is presented.
 (C) Outgrowth of rat E13 commissural neurons on a substrate of AP-TSR-HIS and laminin proteins.
 (D) Commissural neurons grown on F-spondin stained with the TUJ-1 mAb. The focus is on the boundary between F-spondin and BSA substrates, demonstrating that there is no outgrowth on BSA. Scale bar, 200 μ m.
 (E) Commissural neurons grown on F-spondin stained with the anti-TAG-1 mAb 1C12. Scale bar, 50 μ m.
 (F) Commissural neurons grown on F-spondin stained with the anti-DCC mAb. Scale bar, 50 μ m.
 Scale bar values given for bar shown in (F).

underlying them. Growth cones of the commissural axons were shown to cross the ventral midline between the floor plate and the underlying basal membrane (Yaginuma et al., 1991; Bernhardt et al., 1992). To explore the localization of F-spondin protein at the embryonic floor plate, we used the R5 antiserum to stain sections of E4 chick embryos (R2 failed to detect the protein in immunohistochemistry [Burstyn-Cohen et al., 1998]). F-spondin was found in the paranotochordal area, in the extracellular matrix underlying the floor plate, between the floor plate cells and the meninges (Figures 2C and 2D), and in the extracellular matrix surrounding the peripheral nerve (Burstyn-Cohen et al., 1998) (Figures 2C and 2D). Since the chick F-spondin is expressed in both the floor plate and the notochord, it is hard to distinguish between the notochord-derived F-spondin, localized under the pial surface, and the floor plate-derived F-spondin, localized above the pial surface. To verify the precise localization of the floor plate-derived F-spondin, we analyzed the distribution of the protein in rat embryos, since in rodents, F-spondin is not expressed in the notochord (Klar et al., 1992a). To explore further the interaction between F-spondin protein and

the axons, we double labeled rat embryos with the R5 and the monoclonal HNK-1 Ab. In E14 rat embryos, F-spondin immunoreactivity was found under the floor plate in contact with the commissural fibers as they cross the floor plate (Figure 2E). Similarly, in E16 rat embryos, F-spondin immunoreactivity was found under the floor plate and above the pial surface (Figure 5F). Thus, spinal cord-derived F-spondin is deposited in the basal lamina that underlie the floor plate. Its localization implies that it might be involved in the guidance of commissural axons at the floor plate.

In the rostral part of the CNS, the midbrain and the hindbrain, F-spondin immunoreactivity was evident in the neuroepithelium, lateral to the floor plate (Figure 2G). The localization of F-spondin in the ventral lateral neuroepithelium of the hind and midbrain suggests a different role for the protein in these regions (namely, chemoattraction of the commissural neurons or chemorepulsion of the cranial motor neurons). Alternatively, F-spondin Abs may detect, in immunohistochemistry, only the regions that accumulate high levels of the protein, and thus F-spondin is better detected in the rostral CNS, which expresses higher levels of F-spondin than the trunk region.

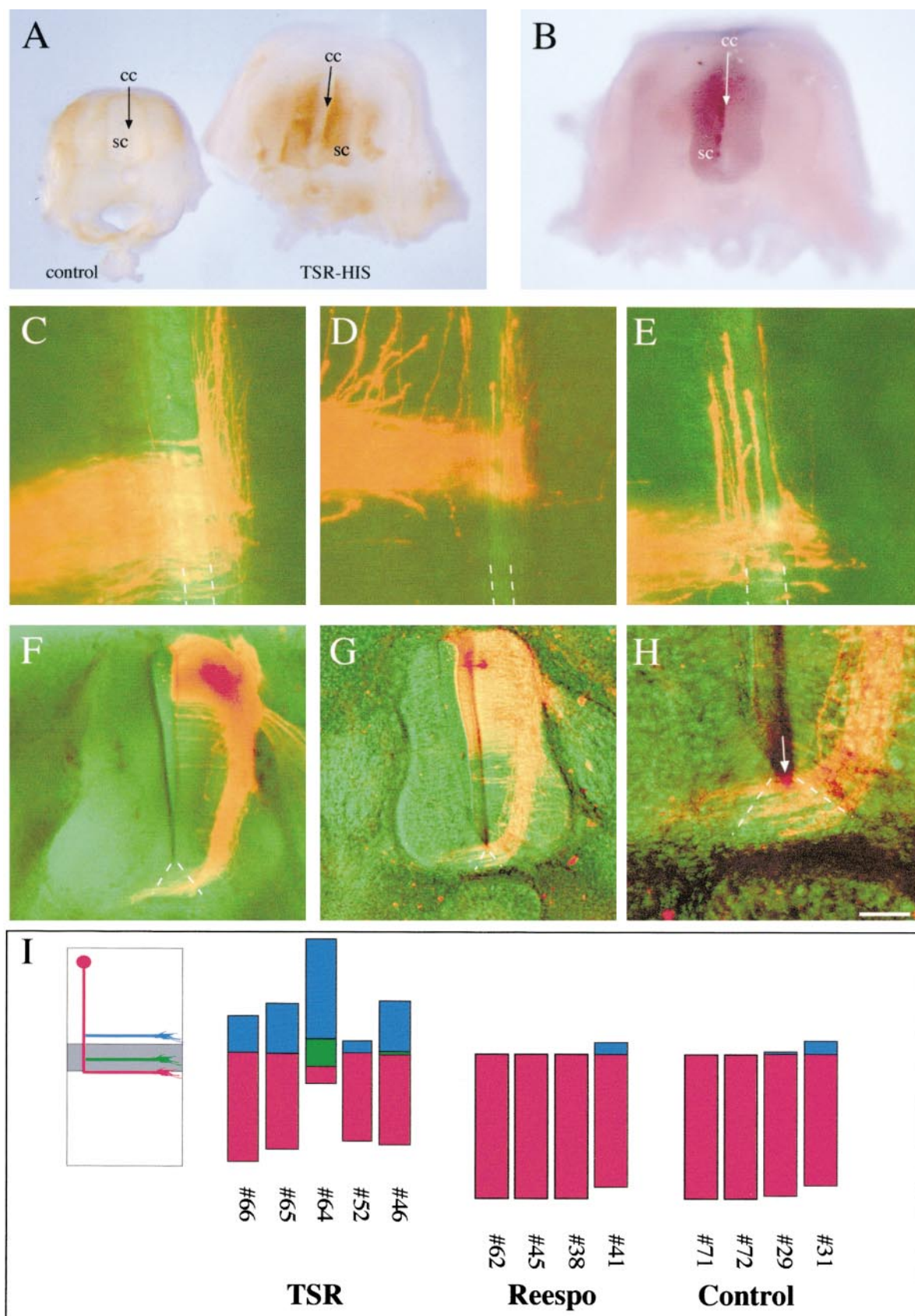


Figure 5. Injection of F-Spondin into the Embryonic Chicken Spinal Cord In Ovo Alters the Trajectories of Commissural Axons
Purified domain proteins were used in an in vivo perturbation assay. The proteins were injected repeatedly into the central canal (arrow, cc) of the spinal cord (sp) in ovo (Stoeckli and Landmesser, 1995). After 2 days, the trajectory of the commissural neurons was analyzed by unilateral injection of Dil into the area of the cell bodies of an open book preparation of the spinal cord.
(A) Embryo injected with biotinylated TSR-HIS protein (right). The injected protein was visualized by streptavidin-peroxidase. The injected protein is deposited in the spinal cord, with higher levels accumulating in the ventricular zone. No staining is observed in uninjected embryo (left). Scale bar, 150 μ m.

Commissural Neurons Bind to the TSR but Not the Reespo Domain Protein

To test whether commissural neurons express a receptor(s) capable of binding F-spondin, we fused the coding regions of both the reespo (AP-reespo) and TSR (AP-TSR) domains of F-spondin to alkaline phosphatase (AP), a readily detectable histochemical label (Figure 3A) (Flanagan and Leder, 1990), and expressed the resulting chimeric proteins in HEK 293 cells. The proteins could be detected by Western blotting of conditioned medium from transfected cells as bands of 120 kDa and 110 kDa, respectively (Figures 3B and 3C). This is consistent with the sizes of the reespo and TSR domains of F-spondin when combined with AP. When medium containing AP-TSR was applied to dissociated cultures of rat E14 dorsal spinal cord neurons, AP reactivity could be detected on the cell bodies, neurites, and growth cones of the cultured neurons (Figures 3D and 3E). In contrast, cultures incubated with the AP-reespo fusion protein and control cultures incubated with secreted alkaline phosphatase (SEAP), also expressed in HEK 293 cells, showed no detectable binding (Figures 3F and 3G; SEAP data not shown). We conclude that the putative receptor of F-spondin is expressed on commissural neurons and binds to the TSR domain of the protein.

The TSR Domain of F-Spondin Promotes the Outgrowth of Dissociated Commissural Neurons In Vitro

F-spondin was shown to be a potent outgrowth-promoting substrate for sensory and hippocampal neurons (Burstyn-Cohen et al., 1998; Y. F. et al., unpublished data). Furthermore, F-spondin is localized to the ECM underlying the floor plate at the time when commissural axons cross the floor plate. To test whether F-spondin could also promote the outgrowth of commissural neurons, we generated fragments of F-spondin corresponding to specific domains with an epitope tag: reespo-HIS, TSR-HIS, and AP-TSR-HIS, containing a *myc* tag and a cassette of six histidines fused to the reespo domain, the TSR domain, and the AP-TSR protein, respectively. The cDNAs encoding these fragments or the full-length protein, F-spondin-HIS (Burstyn-Cohen et al., 1998),

were cloned into a mammalian expression vector and transfected into HEK 293 cells. The conditioned medium of transfected HEK 293 cells was subjected to affinity purification (Figure 4A), and the recombinant proteins were immobilized on nitrocellulose according to Lemmon et al. (1989) to generate substrates for neurite outgrowth assays. Laminin and bovine serum albumin (BSA) were coated similarly as control substrates. A suspension of single neurons of rat E13 dorsal spinal cord was plated onto these substrates, and neurite outgrowth was measured after 40 hr. Neurons cultured on BSA (Figure 4D) and reespo-HIS (data not shown) did not adhere to the substrate proteins. Neurons cultured on F-spondin-HIS, TSR-HIS, and AP-TSR-HIS extended long neurites (Figures 4B–4D), comparable to the extent of neurite growth on laminin (Figure 4C). The extent of outgrowth on F-spondin-HIS, TSR-HIS, and AP-TSR-HIS was similar (Figures 4B and 4C). The majority of the neurites grown on F-spondin (>95%) expressed TAG-1 (Figure 4E) and DCC (Figure 4F) on their surface, supporting the assumptions that the neurons extending axons were indeed commissural neurons. The outgrowth-promoting activity of the F-spondin domains correlates with their axon-binding capabilities; the TSR domain binds to commissural neurons and promotes their outgrowth, whereas the reespo domain neither binds nor promotes outgrowth. Therefore, we conclude that the activity of F-spondin, after its proteolytic processing, is attributed to the TSR domain protein. However, the in vitro data demonstrate that the proteolytic processing is not required for the activation of F-spondin.

Injection of F-Spondin into the Central Canal of Embryonic Chicken Spinal Cord In Ovo Alters the Trajectories of Commissural Axons

F-spondin's pattern of expression together with our finding that secreted F-spondin can promote neurite outgrowth of commissural neurites when used as a substrate suggest that F-spondin may play a role in the guidance of commissural axons at the floor plate. To test this hypothesis in vivo, purified fragments and full-length F-spondin proteins were used in an in vivo perturbation assay. The proteins were injected repeatedly into

(B) Embryo injected with the AP-TSR-HIS protein. Scale bar, 150 μ m.

(C) Uninjected embryo. The axons reach the contralateral side of the floor plate and turn rostrally along the floor plate boundary. Scale bar, 50 μ m.

(D and E) Embryos injected with the TSR-HIS protein. Many axons failed to cross the midline and turned longitudinally either before crossing or within the floor plate. Scale bar, 50 μ m.

(F) Transverse section of the spinal cord of an uninjected embryo. The commissural axons are crossing under the floor plate as a tight bundle. Scale bar, 100 μ m.

(G) Transverse section of the spinal cord of a TSR-injected embryo. The commissural axons do not fasciculate as they approach the floor plate, and they cross the floor plate through its entire height. Scale bar, 100 μ m.

(H) High magnification of (E), showing that some of the axons are reaching the ventral central canal (arrow). Scale bar, 25 μ m.

(I) Quantification of the percentage of axons committing pathfinding errors. NIH image software was used to measure the total area occupied by axons (Dil-positive) at the ipsilateral side (blue), the contralateral side (red), and in the floor plate (green). The bars represent the proportion (percentage) of axons turning in each domain (ipsilateral floor plate, floor plate, and contralateral boundary) for each embryo. For embryos analyzed at several injection points, the sum of the proportions of the injected sites is presented.

The proportions of the TSR and the reespo groups were compared with the control group. The Wilcoxon sign rank test (Siegal, 1956) reveals a significant difference between the TSR and the control group ($p = 0.0008$). However, no significant difference was found between the reespo and the control group ($p = 0.2016$).

The broken lines in (C) through (H) represent the floor plate boundaries. Scale bar values given for bar shown in (H).

Table 1. Injection of F-Spondin into the Embryonic Chicken Spinal Cord In Ovo Alters the Trajectories of Commissural Axons

Embryo	Protein	IL (%)	FP (%)	CL (%)
46a	TSR	29.79	5.28	64.92
46b	TSR	37.30	1.76	60.93
46c	TSR	71.47	4.65	23.87
46d	TSR	24.67	0	75.32
46e	TSR	12.39	0	87.60
52b	TSR	23.97	0	76.02
52c	TSR	8.20	0	91.79
52e	TSR	3.33	0	96.66
64b	TSR	68.49	19.48	12.01
65a	TSR	34.39	0	65.60
66b	TSR	25.38	0	74.61
41b	reespo	0	0	100
41c	reespo	0	0	100
41e	reespo	22.69	0	77.30
45b	reespo	0.03	0	99.96
45a	reespo	0	0	100
62a	reespo	0	0	100
62b	reespo	0	0	100
38c	reespo	0	0	100
38e	reespo	0	0	100
71c	UN	0	0	100
71d	UN	0.39	0	99.60
72b	UN	0	0	100
72a	UN	0.86	0	99.13
31a	NRS	8.16	0	91.83
29b	NRS	1.93	0	98.06
29a	NRS	0	0	100

Purified domain proteins were used in an in vivo perturbation assay. The proteins were injected repeatedly into the lumen of the spinal cord in ovo (Stoeckli and Landmesser, 1995). Quantification of the proportion of axons turning at the ipsilateral floor plate (IL), in the floor plate (FP), or at the contralateral boundary (CL) was performed by using NIH image software.

the lumen of the spinal cord in ovo. After 2 days, the trajectory of the commissural neurons was analyzed by unilateral injection of 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (Dil) into the cell body area (Stoeckli and Landmesser, 1995). To examine the distribution of the injected protein, two forms of purified tagged proteins were injected: a biotinylated TSR domain protein and an AP-TSR-HIS fusion protein. The tagged proteins were detected by avidin-peroxidase and AP reactivity, respectively. In both cases, the injected tagged proteins were detected through the entire neuroepithelium, with higher levels deposited in proximity to the central canal, namely, in the ventricular zone (Figures 5A and 5B). Thus, the injected proteins are distributed ectopically throughout the entire spinal cord. The injection of TSR-HIS (Figures 5D, 5E, and 5I) and F-spondin-HIS (data not shown) proteins caused pathfinding errors of commissural axons. Many axons failed to cross the midline and turned longitudinally either before crossing the floor plate or within it. Pathfinding errors were evident in most of the TSR-HIS injections (ten of eleven) (Table 1). To quantify the percentage of axons performing pathfinding errors, we used an NIH image software package to measure the total area occupied by axons (Dil-positive) at the ipsilateral side, the contralateral side, and in the floor plate. For the TSR-HIS-injected embryos, the range

of pathfinding errors varied from 68% ipsilateral, 19% within the floor plate, 12% contralateral in the most extreme case to 8% ipsilateral, 92% contralateral in the least affected case (Figures 5D, 5E, and 5I; Table 1). Reespo-HIS did not appear to have any effect on commissural axon pathfinding, as in eight of nine cases, the Dil-injected site had normal trajectories, and in one of nine, 22% of the axons turned ipsilaterally (Figure 5I; Table 1). This was similar to control embryos that were either injected or not injected with nonimmune rabbit IgG; contralateral turns were found in six of seven cases, and in one case, 8% of the axons turned along the ipsilateral border of the floor plate (Figures 5C and 5I; Table 1).

The F-spondin and TSR domain proteins were injected into the central canal. As shown, high concentrations of the proteins are associated with the ventricular zone (Figures 5A and 5B). Attraction toward the ventricular zone should be evident as ipsilateral turning of the axons in an open book preparation. To this end, we have prepared transverse sections of manipulated embryos. In the uninjected embryo, the axons fasciculate as they approach the floor plate and cross the floor plate as a tight bundle underneath the floor plate cells (Figure 5F). In the TSR-injected embryo, the axons do not fasciculate; they cross the floor plate through its entire height (Figures 5G and 5H), and many of the axons reach the ventral central canal (Figure 5H). Thus, the injected F-spondin does appear to be attracting the commissural axons.

The erroneous turning of the commissural axons is accompanied by defasciculation. The ectopic F-spondin probably shifts the balance between intrinsic (axonal) and extrinsic (substrate) factors. Thus, the axon would prefer to grow on the F-spondin substrate and will defasciculate.

Blocking the Function of F-Spondin In Ovo Causes Abnormal Turning Behavior of Commissural Axons at the Floor Plate Exit Site

The ectopic F-spondin phenotype does not discriminate between the possible activities of F-spondin as an attractant. It may act to attract the commissural axons to the floor plate, or it may be required as a contact-mediated attraction molecule at the floor plate. Alternatively, it may attract the commissural axons to the contralateral boundary of the floor plate. Blocking the endogenous F-spondin would elucidate its precise nature of activity. Therefore, we tried to block F-spondin in ovo by injecting domain-specific Abs into the embryonic chicken spinal cord. It was found that Abs against the TSR domain (R2) were more effective, producing more aberrant phenotypes, than Abs against the spondin domain (R5). In most of the R2-injected embryos (five of six), all the axons reached the contralateral side of the floor plate. However, the axons' turn into the longitudinal axis along the contralateral border of the floor plate appeared very disorganized. Instead of a smooth and orchestrated maneuver, the axons turned individually, and many drifted away or overshot laterally, resulting in a defasciculated growth pattern along the contralateral border. (Figures 6A and 6B; Table 2). For a quantitative analysis of the observed changes in turning behavior, we measured the

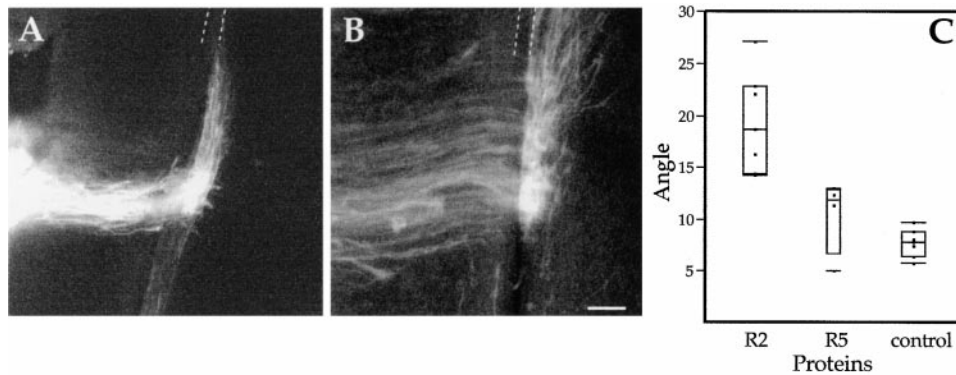


Figure 6. Blocking the Function of F-Spondin In Ovo Causes Abnormal Turning Behavior of Commissural Axons at the Floor Plate Exit Site. Abs (10 mg/ml) against the reespo domain (R5) or against the TSR domain (R2) were injected repeatedly into the lumen of the spinal cord in ovo.

(A) Injection of the NRS. The axons reach the contralateral side of the floor plate and turn rostrally along the floor plate boundary. Scale bar, 100 μ m.

(B) Injection of the R2 Abs. The axons defasciculate at the contralateral side of the floor plate. Scale bar, 100 μ m.

(C) Quantitative analysis of the observed changes in turning behavior. The angle between the floor plate border and the laterally projecting axons was measured. A comparison of the angles obtained from the R2- and the R5-injected embryos with the control group, using Dunnett's method (Dunnett et al., 1955), shows a significant difference between the R2 group and the control group, and no difference between the R5 group and the control group, under a significant level of $\alpha = 0.05$.

Scale bar values given for bar shown in (B).

angle between the floor plate border and the laterally projecting axons. In control embryos, the angle ranged from 5 to 10 (average, $7.75^\circ \pm 1.35$), whereas in the R2-injected embryos, the angle was three to four times wider, ranging from 12 to 23 (average, $19.39^\circ \pm 4.84$). The anti-spondin domain Ab R5 had a modest but not significant effect (average, $10.48^\circ \pm 3.65$) (Figure 6C).

The aberrant turns at the contralateral side of the floor plate suggest that F-spondin is required to keep the longitudinal axons along the floor plate boundary, thus

supporting the hypothesis that F-spondin acts locally, at the floor plate, to attract commissural axons to the contralateral side of the midline.

Discussion

In the current study, we describe the cloning of chick F-spondin. We have shown that, like the rodent, *Xenopus*, and zebrafish F-spondins, the avian gene is also expressed in the embryonic floor plate at a time when commissural axons project toward and across the floor plate. The F-spondin protein is proteolytically processed into 60 kDa fragments consisting of the reespo domain and into 40 kDa and 50 kDa fragments consisting of five or six TSRs, respectively. Immunohistochemistry with an F-spondin-specific Ab shows that the protein accumulates in the basal lamina that underlie the floor plate. The recombinant TSR domain but not the reespo domain of F-spondin binds to the commissural axons and promotes their outgrowth in vitro. In ovo perturbation experiments with either purified proteins or function-blocking Abs caused pathfinding errors and/or aberrant turns at the contralateral border of the floor plate, verifying that F-spondin is required for commissural axon pathfinding at the floor plate.

Various Neuronal Subtypes Respond Differentially to the Reespo and TSR Domains of F-Spondin

We had previously demonstrated that F-spondin and mindin, a structurally related secreted protein that contains a spondin domain and one TSR, promote outgrowth of sensory and hippocampal neurons (Burstyn-Cohen et al., 1998; Y. F. et al., unpublished data). In our previous study (Klar et al., 1992a), we observed outgrowth of commissural axons on purified F-spondin, but the background outgrowth on the controls was too

Table 2. Blocking the Function of F-Spondin In Ovo Causes Abnormal Turning Behavior of Commissural Axons at the Floor Plate Exit Site

Embryo	Ab	Angle
71c	UN	8.81
71d	UN	5.77
72b	UN	9.71
72a	UN	7.9
31a	NRS	8.15
29b	NRS	7.52
29a	NRS	6.4
19d	R2	18.69
19e	R2	14.42
19c	R2	27.14
20a	R2	22.91
3a	R2	22.05
18b	R2	16.3
18a	R2	14.25
24a	R5	12.4
25a	R5	5.09
27e	R5	11.41
26a	R5	13.02

Abs (10 mg/ml) against the reespo domain (R5) or against the TSR domain (R2) were injected repeatedly into the lumen of the spinal cord in ovo. The angle between the floor plate border and the laterally projecting axons was measured. Abbreviations: UN, uninjected, and NRS, normal rabbit IgG.

high to evaluate the contribution of the F-spondin protein. In the current study, we improved the purification procedure, and hence, no background was observed in any of the control experiments.

In vitro experiments with either domain-specific F-spondin Abs or domain-specific mindin Abs demonstrate that the spondin domain of mindin and the reespo domain of F-spondin promote the outgrowth of sensory neurons. In contrast, hippocampal neurons did not extend neurites on the spondin domain of mindin, but did so on the TSR (Y. F. et al., unpublished data). To examine the relative contribution of each domain of F-spondin, we generated recombinant proteins consisting of either the reespo or the TSR domain. In the current study, we provide evidence that like hippocampal neurons, the commissural axons require the TSR domain for outgrowth in vitro. These findings are consistent with the results of the in ovo perturbation experiments. In vivo injections of either the TSR domain, the full-length F-spondin, or the anti-TSR-specific Ab R2 resulted in pathfinding errors, whereas the injection of the reespo domain or the anti-reespo domain-specific Ab R5 did not have a significant effect.

This current in vitro assay and our previous results demonstrate that F-spondin promotes the outgrowth of various neuronal subpopulations. Unlike the neuronal F-spondin, in avian, the somite-derived F-spondin is an inhibitory signal involved in patterning the segmental migration of neural crest cells and their topographical segregation within the rostral somite domain (Debby-Brafman et al., 1999). Thus, F-spondin seems to mediate both negative as well as positive activities, depending on its cellular context and on the identity of the target cell. Growing evidence points to similar dichotomies in the bioactivities of other molecules implicated in the regulation of neurite extension. For instance, semaphorin D was found to repel cortical axons, while semaphorin E appears to act as a chemoattractive signal (Bagnard et al., 1998). The best-characterized example of dual activity at present is provided by netrin-1, which was shown to act as a chemoattractant for commissural neurons and as a chemorepellent for trochlear motor axons (Kennedy et al., 1994; Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995). In these cases, it was suggested that attractive and repulsive activities are mediated by different receptors (reviewed by Tessier-Lavigne and Goodman, 1996).

Mode of Action of F-Spondin

The trajectory of the commissural axons toward and across the ventral midline of the CNS is mediated by a variety of evolutionary conserved attractive and repulsive signals. Both in vitro and in vivo experiments demonstrated that the invertebrate as well as the vertebrate netrin-1 are mediating the attraction toward the ventral midline of the CNS (Hedgecock et al., 1990; Ishii et al., 1992; Harris et al., 1996; Mitchell et al., 1996; Serafini et al., 1996). It should be noted that evidence also exists for the participation of other factors in the attraction close to the floor plate. First, the embryonic brain contains a synergizing activity for netrin-1 (Serafini et al., 1994). Second, a floor plate chemoattractive activity that orients commissural axons toward the floor plate in a

turning assay is not blocked by Abs to the netrin-1 receptor DCC (Keino-Masu et al., 1996). Because a gradient of netrin would be sufficient for attracting axons over longer distances, high and possibly saturating levels near the floor plate might prevent growth cones from detecting the gradient. Thus, with respect to short distance guidance at the floor plate or in the ventral horn, additional cues directing axons across the midline might be required.

Based on our previous studies, we hypothesize that F-spondin may guide the commissural axons in any of the following ways: (1) as a diffusible chemoattractant guiding the commissural axons toward the floor plate; (2) as a contact-mediated attractant molecule required for crossing the floor plate; (3) as a contact-mediated repellent molecule preventing recrossing of the floor plate; or (4) as a short-range attractant with the biological activity of reattracting the axons to the floor plate at its contralateral boundary.

The chemoattractant hypothesis is supported by the behavior of the commissural axons in the protein injection experiments. However, experiments in which dorsal spinal cord explants were challenged with recombinant F-spondin failed to demonstrate any attraction toward F-spondin or any synergizing activity for netrin-1 (T. B.-C., A. K., and M. Tessier-Lavigne, unpublished data). However, it is conceivable that in vivo, the diffusion of F-spondin is mediated by factors that are absent in the in vitro assay or that interactions with other factors expressed in the spinal cord are required to exert its activity. It should be noted that similar in ovo experiments with the netrin-1 protein resulted in a more pronounced phenotype; almost all of the commissural neurons turned to the central canal (E. S. and M. Tessier-Lavigne, unpublished data). Therefore, we suggest that netrin-1 is a long-range chemoattractant, while F-spondin acts as a short-range attractive cue. The fact that the commissural axons reached the floor plate in the Ab blocking experiments in ovo suggests that netrin-1 is sufficient for attracting commissural axons to the floor plate and that the attractive activity of F-spondin is required only locally.

The in vitro outgrowth data demonstrated that F-spondin supports the outgrowth of commissural axons. Hence, it is reasonable to speculate that F-spondin, which is presented to the commissural growth cones as they encounter the floor plate, acts as a contact-mediated attractant molecule that supports outgrowth at the floor plate. The in vivo blocking experiments do not support this assumption. However, it is conceivable that we haven't blocked completely the activity of the endogenous F-spondin and that the amount of the unblocked protein is sufficient to allow crossing the floor plate. Alternatively, F-spondin is not the only adhesion molecule expressed in the floor plate. It is likely that F-spondin acts in concert with other floor plate adhesion molecules in mediating the contact attractant activity of the floor plate.

The in vitro outgrowth data and the in vivo protein injection contradict the hypothesis that F-spondin acts as a contact-repellent molecule. Formally, an additional receptor that mediates repulsion might be expressed as the axons cross the floor plate, and this receptor may not be expressed on the cultured neurons. In that case,

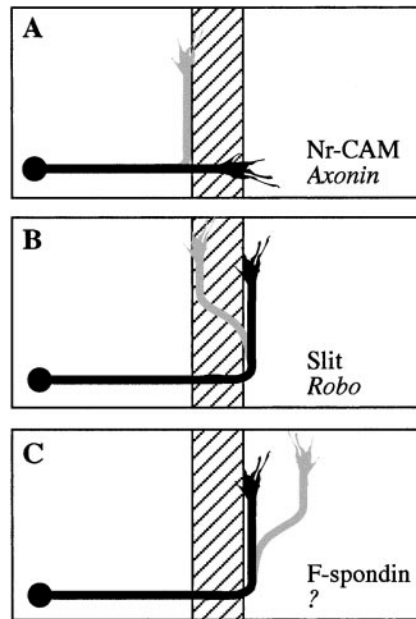


Figure 7. Interaction between the Floor Plate Surface, the Matrix Molecules, and the Receptors Expressed on Commissural Axons (A) NrCAM is required at the ipsilateral floor plate boundary in order for commissural axons to enter the floor plate. NrCAM mediates its activity by binding to axonin-1 expressed on the commissural axons. (B) At the contralateral side of the floor plate, Slit is required to prevent commissural axons from recrossing the floor plate. Slit binds to the Robo receptor expressed by commissural axons after contact with the floor plate. (C) At the contralateral side of the floor plate, F-spondin is required to maintain the commissural axons along the floor plate boundary. F-spondin activity is mediated by an as yet unidentified receptor. The black lines represent the wild-type trajectory. The gray lines represent the aberrant trajectory after inactivation of NrCAM (Stoeckli et al., 1995) and F-spondin in ovo and the null phenotype of Robo and Slit in the fly (Kidd et al., 1998a, 1999).

blocking the endogenous F-spondin should result in commissural axons recrossing the floor plate after they reach the contralateral floor plate boundary. The results obtained in the Ab blocking experiments, namely, that commissural axons were deflected away from the contralateral floor plate boundary, do not support the assumptions that F-spondin is acting as a contact-repellent molecule.

Consistent with a role for F-spondin as a short-range attractant that keeps the axons at the contralateral floor plate boundary are the observations made in the in ovo experiments: the turning of the axons toward the injected protein revealed the potential of F-spondin as a short-range attractant. The observed phenotype, after in ovo injection of Abs specific to the TSR domain of F-spondin, suggests that F-spondin is required for attracting the commissural axons at the contralateral side of the floor plate.

To maintain the longitudinal fascicle along the floor plate boundary, two activities are required: first, axons need to be prevented from growing back into the floor plate. This is achieved by the repulsive activity of the Slit protein, which is mediated by the Robo receptor

(Figure 7B). In addition, axons need to be prevented from growing laterally, away from the floor plate, by attracting them back to the floor plate (Figure 7C). The combined push and pull activities of the floor plate restrict the longitudinal trajectory of the commissural axons to the floor plate contralateral boundary. Blocking F-spondin, as was demonstrated in the Ab injection experiment, shifts the balance toward the repulsion from Slit. The repulsion force is probably stronger than the intrinsic axonal adhesion, which causes the axons to defasciculate from the longitudinal bundle and to extend laterally, away from the floor plate. Our results suggest that F-spondin is providing the activity that is required for attracting the commissural axons back to the floor plate border.

F-Spondin Acts Locally to Guide Commissural Axons into the Longitudinal Axis

Previous in vivo and in vitro experiments (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997) suggested that, similar to the situation in invertebrates (Seeger et al., 1993; Kidd et al., 1998a, 1998b), a balance between positive and negative signals would determine the behavior of axons at the midline of the vertebrate CNS (Figure 7). NrCAM expressed on the floor plate surface is involved in enhancing the adhesion between the growth cone and the floor plate. Because F-spondin is secreted by the floor plate, its mode of action may differ from that of NrCAM. The in vitro outgrowth assay presented in this study demonstrates that in vitro F-spondin promotes outgrowth of commissural axons. Thus, it could be predicted that perturbing F-spondin levels would result in a phenotype similar to the one seen in the axonin/NrCAM perturbation experiments, namely, early turning at the ipsilateral floor plate boundary (Stoeckli and Landmesser, 1995). The different trajectory of the commissural axons in the F-spondin perturbation experiments, the attraction toward the ectopic F-spondin, and the lateral drifting at the contralateral side of the floor plate in the blocking experiments suggest that NrCAM is required and sufficient for the correct "entering" into the floor plate (Figure 7A), while F-spondin is required at the "exit point," the contralateral side of the floor plate, to maintain the longitudinal trajectory along the floor plate boundary (Figure 7C).

Experimental Procedures

Cloning of the Chick F-Spondin

An E2.5 chick spinal cord library was screened with the full-length rat *F-spondin* cDNA. The sequence of the gene is available from GenBank (accession number AF149302).

DNA Constructs

To generate the pR8 plasmid (for expressing amino acids 22–142 of the rat F-spondin), the fragment between the PCR primer AGAAG ATCTGCGCTCGCTTCTCGGATGA (amino acid 22) and the BamHI site (amino acid 142) was subcloned into the pQE-32 vector (Qiagen).

To generate the pR3 plasmid, the NheI fragment (from nucleic acid 1834 [amino acid 567] to nucleic acid 2743 [in the 3' untranslated region]) of the chick F-spondin was subcloned into the pQE-32 vector (Qiagen).

To generate the pSecTS0 plasmid for expressing the pReespo-HIS domain, the primers GCAAGCTTGCCTCGCTTCTCGGATG and GAGAGATCTAGGGGTGTCATCTTCATC were used to synthesize a PCR fragment corresponding to the region of amino acids

22–440 of the rat F-spondin. The PCR product was subcloned into the HindIII and Xba sites of pSecTagB (Invitrogen).

To generate the pSec1–5a for expressing the TSR–HIS domain protein, the primers GCAAGCTTTGCATCTACTCCAAGTGGTC and CGTCTAGAGAACTGCTCTCCATCTGAC were used to synthesize a PCR fragment corresponding to the region of amino acids 443–752 of the rat *F-spondin* gene. The PCR product was subcloned into the HindIII and Xba sites of pSecTagB (Invitrogen).

To generate the AP–reespo fusion protein, the region corresponding to amino acids 22–440 of the rat F-spondin underwent PCR (see pSecTS0). The PCR fragment was ligated to a HindIII–BgIII AP fragment of the pAPtag4 plasmid, and the ligated product was inserted into a pcDNA3 expression vector (Invitrogen) previously cut with HindIII and XbaI.

To generate the AP–TSR–HIS fusion protein, a fragment containing the TSR repeats 1–5 was PCR'd from the pSec1–5a plasmid by the use of the primer corresponding to amino acid 443, GCAAGCTTGCATCTACTCCAAGTGGTC, and the primer CGGCTAGCTAGAA GGCACAGTCGAGG, corresponding to the pSecTagB sequence 3' to the *myc*–HIS cassette. The fragment was cut with BgIII and NheI and ligated into pcDNA–SEAP.

Production of Abs

Production of the R2 and R5 Abs was previously described (Burstyn-Cohen et al., 1998). The R3 and R8 Abs were produced in a similar way. The plasmids encoding the relevant fragments were introduced into *Escherichia coli*, the expression of the proteins was induced by isopropyl- β -D-thiogalactopyranoside, and the recombinant proteins were purified by adsorption on a Ni–NTA (Qiagen) column, according to the manufacturer's directions. The purified proteins were injected into rabbits (250 μ g protein/injection in adjuvant; total of three injections), and the sera were tested for immunoreactivity on Western blots and for immunohistochemistry on Bouin's fixed transverse sections of embryonic tissue.

Immunohistochemistry

Chick embryos at the designated stages were fixed in Bouin's fixative. Tissues were dehydrated and embedded in paraffin. Sections (7 μ m thick) were cut, mounted on Superfrost Plus (Fisher Scientific) slides, rehydrated, and incubated overnight in a humidified chamber at 4°C with a 1:1000 dilution of the appropriate antiserum diluted in blocking solution (5% goat serum in phosphate-buffered saline [PBS]). The sections were washed twice in PBS, incubated with FITC- or Cy2-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch; diluted 1:200 in blocking solution) in a humid chamber for 2 hr at room temperature, rinsed twice in PBS, mounted in Fluoromount, and examined by indirect immunofluorescence on a Zeiss Axiovert.

Neurite Outgrowth Assays

The dorsal parts of E14 rat spinal cords were dissected and incubated with 0.025 mg/ml trypsin (Gibco) for 20 min in a Ca^{2+} /Mg $^{2+}$ -free modified essential medium (S-MEM; Gibco) supplemented with 8 mg/ml glucose. The tissue was then washed with S-MEM; triturated to a single cell suspension; plated at a density of 100,000 cells/35 mm diameter tissue culture dish on appropriate substrates in Ham's F12 medium (Gibco) supplemented with N3 (F12–N3), glutamine, GlutaMAX I (Gibco–BRL), MEM vitamins (Kibbutz Beit Haemek, Israel), 5% heat-inactivated horse serum, and antibiotics; and placed in a 5% CO $_2$ -humidified incubator at 37°C. F-spondin–HIS, reespo–HIS, TSR–HIS, and AP–TSR–HIS were affinity purified on a Talon affinity column (Clontech). Affinity-purified proteins were absorbed onto nitrocellulose (Lemmon et al., 1989). The nitrocellulose was then blocked with BSA (30 mg/ml), which provided a further control for background neurite outgrowth. E14 dorsal spinal cord neurons were plated on immobilized protein substrates at a density of 100,000 cells/35 mm diameter dish and grown for 48 hr. Cultures were then fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with either anti-tubulin III mAb TUJ-1, anti-TAG-1 mAb 1C12 (Dodd et al., 1988), or the anti-DCC mAb (Keino-Masu et al., 1996), which can recognize proteins expressed specifically by commissure axons. Neuronal cell bodies and neurites were

visualized by indirect immunofluorescence on a Zeiss Axioplan microscope. Neurite lengths were measured as the distance from the edge of the soma (sharply defined by the obtained fluorescence) to the tip of its longest neurite. Neurite lengths were only measured if the entire length of neurite could be unambiguously identified.

Protein Extracts and Western Blotting

Proteins were extracted from E4 or E5 chick spinal cords with RIPA buffer in the presence of protease inhibitors. Equal amounts of protein (4 μ g) were loaded onto SDS–PAGE and transferred onto nitrocellulose membranes. Western blots were incubated with R3 or R8 IgG fractions, followed by horseradish peroxidase–(HRP–) conjugated anti-rabbit secondary Abs. An enhanced chemiluminescence reaction resulted in visualization of the protein bands.

In Ovo Perturbation Assays

All procedures followed those of Stoeckli et al. (1995), with slight modifications. The purified recombinant domain-specific protein was dialyzed against F12 to remove any traces of Tris or salts. Intraluminal injection was executed with a 10 mg/ml IgG fraction of the normal rabbit serum (NRS) control Ab or the anti-F-spondin R2 or R5 Abs. Domain-specific proteins were injected at the following concentrations: 0.45 mg/ml of reespo–HIS and 0.25 mg/ml of TSR–HIS.

AP Binding Experiments

For binding to rat E14 dorsal spinal cord culture, cells were dissociated as described above and plated at a density of 200,000 cells/35 mm diameter dish precoated with poly-D-lysine and laminin. The cells were cultured at 37°C in 5% CO $_2$ for 20 hr in the same medium used for the outgrowth assay. After incubation with conditioned media containing TSR–AP or AP, cells were processed for AP histochemistry as described (Cheng and Flanagan, 1994).

Visualization of Injected Proteins

AP–TSR–HIS and TSR–HIS proteins were purified as described. TSR–HIS was biotinylated by using the Boehringer Mannheim Biotin Labeling Kit according to the manufacturer's instructions. Injected embryos were cut transversely in 400–500 μ m sections and processed as small whole-mount pieces, as follows. Biotinylated TSR–HIS and control embryos were blocked in 2% BSA in PBS overnight at 4°C with gentle agitation, followed by PBS washes. Endogenous HRP was inactivated in 0.3% H $_2$ O $_2$ for 30 min at room temperature, incubated with streptavidin–HRP (Boehringer Mannheim) in a 1:1000 dilution in 2% BSA/PBS for 1 hr at room temperature, and washed several times in PBS. HRP reactivity was developed with diaminobenzidine as a substrate. Color reaction was stopped by washing in PBS. For AP–TSR–HIS and control embryos, endogenous AP reactivity was inactivated at 65°C for 100 min. Embryo slices were then equilibrated for 10 min in freshly prepared AP buffer (100 mM Tris [pH 9.5], 100 mM NaCl, and 5 mM MgCl $_2$). AP reactivity was developed by adding 0.34 mg/ml nitroblue tetrazolium and 0.18 mg/ml 5-bromo-4-chloro-3-indolylphosphate toluidinium (Boehringer Mannheim). Color developed in the dark overnight, at room temperature. Photos were taken with a Zeiss Stemi-SR stereoscope.

Acknowledgments

The authors thank Tom Jessell, in whose laboratory the initial research, including the cloning of the chick F-spondin, was performed and who provided constructive suggestions and insights; Marc Tessier-Lavigne and Corey Goodman for sharing their results prior to publication and for suggestions and insights; Chaya Kalcheim and Joel Yisraeli for comments on the manuscript; Chris Cox for sequencing the chick *F-spondin* cDNA; and Kan Yang for constructing the pR8 plasmid. This work was supported by grants to A. K. from the Israel Cancer Research Foundation, the Israel–USA Binational Foundation, the Israel Science Foundation, and Cambridge Neuroscience. E. S. is supported by a grant from the Swiss National Science Foundation.

Received January 19, 1999; revised April 12, 1999.

References

- Bagnard, D., Lohrum, M., Uziel, D., Pueschel, A.W., and Bolz, J. (1998). Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections. *Development* 125, 5043-5053.
- Bernhardt, R.R., Nguyen, N., and Kuwada, J.Y. (1992). Growth cone guidance by floor plate cells in the spinal cord of zebrafish embryo. *Neuron* 8, 869-882.
- Bornstein, P., O'Rourke, K., Wikstrom, K., Wolf, F.W., Katz, R., Li, P., and Dixit, V.M. (1991). A second, expressed thrombospondin gene (Thbs2) exists in the mouse genome. *J. Biol. Chem.* 266, 12821-12824.
- Bovolenta, P., and Dodd, J. (1990). Guidance of commissural growth cones at the floor plate in the embryonic rat spinal cord. *Development* 109, 435-447.
- Bovolenta, P., and Dodd, J. (1991). Perturbation of neuronal differentiation and axon guidance in the spinal cord of mouse embryos lacking a floor plate: analysis of Danforth's short-tail mutation. *Development* 113, 625-639.
- Brose, B., Bland, K.S., Wang, K.H., Arnott, D., Henzel, W., Goodman, C.S., Tessier-Lavigne, M., and Kidd, T. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* 96, 795-806.
- Burstyn-Cohen, T., Frumkin, A., Xu, Y.T., Scherer, S.S., and Klar, A. (1998). Accumulation of F-spondin in injured peripheral nerve promotes the outgrowth of sensory axons. *J. Neurosci.* 18, 8875-8885.
- Cheng, H.J., and Flanagan, J.G. (1994). Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. *Cell* 79, 157-168.
- Colamarino, S.A., and Tessier-Lavigne, M. (1995). The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. *Cell* 81, 621-629.
- D'Arcangelo, G., Miao, G.G., Chen, S.C., Soares, H.D., Morgan, J.I., and Curran, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature* 374, 719-723.
- Debby-Brafman, A., Burstyn-Cohen, T., Klar, A., and Kalcheim, C. (1999). F-spondin, expressed in somite regions avoided by neural crest cells, mediates inhibition of distinct somite domains to neural crest migration. *Neuron* 22, 475-488.
- Dodd, J., Morton, S.B., Karagogeos, D., Yamamoto, M., and Jessell, T.M. (1988). Spatial regulation of axonal glycoprotein expression on subsets of embryonic spinal neurons. *Neuron* 1, 105-116.
- Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.* 50, 1096-1121.
- Fazeli, A., Dickinson, S.L., Hermiston, M.L., Tighe, R.V., Steen, R.G., Small, C.G., Stoeckli, E.T., Keino-Masu, K., Masu, M., Rayburn, H., et al. (1997). Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. *Nature* 386, 796-804.
- Flanagan, J.G., and Leder, P. (1990). The kit ligand: a cell surface molecule altered in steel mutant fibroblasts. *Cell* 63, 185-194.
- Hamburger, V., and Hamilton, H.L. (1951). A series of normal stages in the development of the chick embryo. *J. Embryol. Exp. Morphol.* 88, 49-92.
- Harris, R., Sabatelli, L.M., and Seeger, M.A. (1996). Guidance cues at the Drosophila CNS midline: identification and characterization of two Drosophila Netrin/UNC-6 homologs. *Neuron* 17, 217-228.
- Hatta, K. (1992). Role of the floor plate in axonal patterning in the zebrafish CNS. *Neuron* 9, 629-642.
- Hedgecock, E.M., Culotti, J.G., and Hall, D.H. (1990). The *unc-5*, *unc-6*, and *unc-40* genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in *C. elegans*. *Neuron* 4, 61-85.
- Higashijima, S., Nose, A., Eguchi, G., Hotta, Y., and Okamoto, H. (1997). Mindin/F-spondin family: novel ECM proteins expressed in the zebrafish embryonic axis. *Dev. Biol.* 192, 211-227.
- Holley, J., and Silver, J. (1987). Growth pattern of pioneering chick spinal cord axons. *Dev. Biol.* 123, 375-388.
- Ishii, N., Wadsworth, W.G., Stern, B.D., Culotti, J.G., and Hedgecock, E.M. (1992). UNC-6, a laminin-related protein, guides cell and pioneer axon migrations in *C. elegans*. *Neuron* 9, 873-881.
- Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E.D., Chan, S.S., Culotti, J.G., and Tessier-Lavigne, M. (1996). Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. *Cell* 87, 175-185.
- Kennedy, T.E., Serafini, T., de la Torre, J.R., and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* 78, 425-435.
- Kidd, T., Brose, K., Mitchell, K.J., Fetter, R.D., Tessier-Lavigne, M., Goodman, C.S., and Tear, G. (1998a). Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. *Cell* 92, 205-215.
- Kidd, T., Russell, C., Goodman, C.S., and Tear, G. (1998b). Dosage-sensitive and complementary functions of Roundabout and Commisuresless control axon crossing of the CNS midline. *Neuron* 20, 25-33.
- Kidd, T., Bland, K.S., and Goodman, C.S. (1999). Slit is the midline repellent for the Robo receptor in *Drosophila*. *Cell* 96, 785-794.
- Klar, A., Baldassare, M., and Jessell, T.M. (1992a). F-spondin: a gene expressed at high levels in the floor plate encodes a secreted protein that promotes neural cell adhesion and neurite extension. *Cell* 69, 95-110.
- Klar, A., Jessell, T.M., and Altaba, A.R.I. (1992b). Control of floor plate identity and function in the embryonic nervous system. *Cold Spring Harbor Symp. Quant. Biol.* 57, 473-482.
- Kolodziej, P.A., Timpe, L.C., Mitchell, K.J., Fried, S.R., Goodman, C.S., Jan, L.Y., and Jan, Y.N. (1996). *frazzled* encodes a Drosophila member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. *Cell* 87, 197-204.
- Lawler, J., and Hynes, R.O. (1986). The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. *J. Cell Biol.* 103, 1635-1648.
- Lemmon, V., Farr, K.L., and Lagenaur, C. (1989). L1-mediated axon outgrowth occurs via a homophilic binding mechanism. *Neuron* 2, 1597-1603.
- Li, H.S., Chen, J.H., Wu, W., Fagaly, T., Zhou, L., Yuan, W., Dupuis, S., Jiang, Z.H., Nash, W., Gick, C., et al. (1999). Vertebrate Slit, a secreted ligand for the transmembrane protein Roundabout, is a repellent for olfactory bulb axons. *Cell* 96, 807-818.
- Mitchell, K.J., Doyle, J.L., Serafini, T., Kennedy, T.E., Tessier-Lavigne, M., Goodman, C.S., and Dickson, B.J. (1996). Genetic analysis of *Netrin* genes in *Drosophila*: Netrins guide CNS commissural axons and peripheral motor axons. *Neuron* 17, 203-215.
- Ruiz i Altaba, A., Cox, C., Jessell, T.M., and Klar, A. (1993). Ectopic neural expression of a floor plate marker in frog embryos injected with the midline transcription factor Pintallavis. *Proc. Natl. Acad. Sci. USA* 90, 8268-8272.
- Seeger, M., Tear, G., Ferres-Marco, D., and Goodman, C.S. (1993). Mutation affecting growth cone guidance in *Drosophila*: genes necessary for guidance toward or away from the midline. *Neuron* 10, 409-426.
- Serafini, T., Kennedy, T.E., Galko, M., Mirzayan, C., Jessell, T.M., and Tessier-Lavigne, M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. *Cell* 78, 409-424.
- Serafini, T., Colamarino, S.A., Leonardo, E.D., Wang, H., Beddington, R., Skarnes, W.C., and Tessier-Lavigne, M. (1996). Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* 87, 1001-1014.
- Shirasaki, R., Katsumata, R., and Murakami, F. (1998). Change in chemoattractant responsiveness of developing axons at an intermediate target. *Science* 279, 105-107.
- Siegal, S. (1956). *Nonparametric Statistics for the Behavioral Sciences* (New York: McGraw-Hill).
- Stoeckli, E.T., and Landmesser, L.T. (1995). Axonin-1, Nr-CAM, and Ng-CAM play different roles in the in vivo guidance of chick commissural neurons. *Neuron* 14, 1165-1179.
- Stoeckli, E.T., Sonderegger, P., Pollerberg, G.E., and Landmesser, L.T.

L.T. (1997). Interference with axonin-1 and NrCAM interactions unmasks a floor-plate activity inhibitory for commissural axons. *Neuron* 18, 209–221.

Tessier-Lavigne, M., and Goodman, C.S. (1996). The molecular biology of axon guidance. *Science* 274, 1123–1133.

Tessier-Lavigne, M., Placzek, M., Lumsden, A.G.S., Dodd, J., and Jessell, T.M. (1988). Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature* 336, 775–778.

Umekiya, T., Takeichi, M., and Nose, A. (1997). M-spondin, a novel ECM protein highly homologous to vertebrate F-spondin, is localized at the muscle attachment sites in the *Drosophila* embryo. *Dev. Biol.* 186, 165–176.

Yaginuma, H., Homma, S., Kunzi, L., and Oppenheim, R.W. (1991). Pathfinding by growth cones of commissural interneurons in the chick embryo spinal cord: a light and electric microscopic study. *J. Comp. Neurol.* 304, 78–102.

Zallen, J.A., and Bargmann, C.I. (1998). The conserved immunoglobulin superfamily member SAX-3/Robo directs multiple aspects of axon guidance in *C. elegans*. *Cell* 92, 217–227.

GenBank Accession Number

The *F-spondin* sequence reported in this paper has been submitted to GenBank under accession number AF149302.

Note Added in Proof

The data cited throughout as “Y. F. et al., unpublished data” are now in press: Feinstein, Y., Borrell, V., Garcia, C., Burstyn-Cohen, T., Tzarfaty, V., Frumkin, A., Nose, A., Okamoto, H., Higashijima, S., Soriano, A., and Klar, A. (1999). F-spondin and mindin: two structurally and functionally related genes expressed in the hippocampus that promote outgrowth of embryonic hippocampal neurons. *Development*, in press.